

Original Article

EVALUATION OF ACUTE AND SUB-ACUTE TOXICITY OF A STANDARDIZED POLYHERBAL FORMULATION (HC9): AN *IN VIVO* STUDY

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ABSTRACT

Objective: In the present study, we have performed the acute and sub-acute toxicity of a standardized polyherbal formulation (HC9) in Swiss albino mice.

Methods: In acute toxicity study, the mice were orally administered with different doses (1750 and 2000 mg/kg) of HC9 and monitored for 14 d. In the sub-acute toxicity study, animals received HC9 extract by oral gavage at the doses of 250, 500 and 1000 mg/kg/day ($n=5$ /group/sex) for 28 d. At the end of the study, the animals were sacrificed and evaluated for effect of HC9 on biochemical, hematological and histopathological parameters.

Results: HC9 did not produce any adverse effects in biochemical, hematological, urine and histopathological parameters in mice. HC9 did not induce any adverse effects in terms of mortality and clinical signs in the acute toxicity study. It was well-tolerated by mice up to 2000 mg/kg/body weight. In sub-acute toxicity study, no treatment-related adverse effects were found in the mice upto 1000 mg/kg/day dose. No significant changes were observed in biochemical and hematological parameters as well as histopathology of tissues (liver, kidney, spleen, heart, lung, thymus, adrenal gland, epididymis and testis/ovary) among mice of either sex.

Conclusion: Our results showed that HC9 did not induce any acute and sub-acute toxicity in male and female mice, thereby, suggesting its safety for future clinical application.

Keywords: Acute and sub-acute oral toxicity, HC9, Swiss albino mice.

INTRODUCTION

Traditional polyherbal formulations have been used globally from time immemorial for the treatment and prevention of many non-communicable and chronic diseases [1-4]. Recent research focus has shifted towards the use of herbal medicines due to their diverse biological activities, easy availability, cost effective nature and safe usage [5-7]. Polyphenols and flavonoids present in the medicinal plants have been shown to possess significant anticancer properties [8-10]. Various scientific studies, including ours, have suggested the potential of medicinal plants as anti-cancer drug candidates [8-9, 11-14].

Herbal medicines are traditionally given in the form of polyherbal formulations (PHFs) as each ingredient is supposed to have different pharmacological function [15-17]. Since, PHFs have a combination of compounds, it may be possible that one compound may either potentiate the effect or increase the bioavailability or decrease the toxicity of other pharmacologically active compound(s) [15]. PHFs are usually prescribed to be taken for a longer period and hence may cause adverse effects in the patients, thereby warranting evaluation of their efficacy and safety profile [18-19].

In the present study, we have analyzed the safety of a standardized poly herbal formulation (HC9) [20] that we have previously reported exhibiting significant antioxidant potential and cytotoxic activity in breast cancer cell lines [8]. HC9 is composed of nine medicinal herbs that include *Picrorhiza kurroa*, *Cyperus rotundus*, *Zingiber officinale*, *Cedrus deodara*, *Tinospora cordifolia*, *Holarrhena antidysenterica*, *Swertia chirata*, *Cissampelos pareira* and *Hemidesmus indicus* [8, 20]. We have found *in vitro* as well as *in vivo* that HC9 exhibited significant anticancer activity and immunomodulatory potential (Communicated). In the present paper, we have performed the acute and sub-acute oral toxicity of HC9 in both the sexes of Swiss albino mice to evaluate its toxicity, safety and tolerability profile. The samples were analyzed for hematological, biochemical and histopathological parameters. The studies were done according to the Organization for

Economic Cooperation and Development (OECD) guidelines for testing of chemicals.

MATERIALS AND METHODS

Plant materials and preparation of ethanolic extract

The whole/part of plant materials of HC9 were purchased from Shri Shailya Medi Pharms (Solapur, Maharashtra, India). All the nine plant materials were botanically authenticated and validated and voucher specimens were deposited at the Department of Botany, Agharkar Research Institute and Herbaria of Medicinal Plant Conservation Centre (MPCC), Pune as described previously [8]. The dried plant materials were separately ground to powders.

The formulation was prepared by mixing equal parts (1:1 ratio) of each individual plant material of HC9 and subjected to extraction in ethanol by soxhlet apparatus as described previously [8]. Briefly, the resulting extract was centrifuged at 13000 rpm for 15 min, supernatant was filter-sterilized using swiney filter (pore size, 0.45 μ m) and stored in aliquots at -80 °C until use. The filtrate was standardized by phyto chemical and HPTLC method [20].

Animals and maintenance

Healthy Swiss albino mice, 6-8 w old, of either sex, having body weights in the range of 20 \pm 3 g were procured from the animal house of Bioscience (Pune, Maharashtra, India). They were randomly divided into different experimental groups. The animals were housed in polypropylene cages at an ambient temperature of 21 \pm 3 °C and 30-70% relative humidity, with a 12:12 h light/dark rhythm. Animals were acclimatized to laboratory conditions for at least one week prior to the start of the experiment. They were provided with commercial food pellets (Nutrivet, Pune) and water *ad libitum* unless stated otherwise. The study was approved by the Institutional Ethics Committee (CPCSE Reg. No.258/CPCSE) of the Medical College of Bharati Vidyapeeth University, Pune.

Acute toxicity study

The acute toxicity of HC9 was evaluated in female Swiss albino mice having body weights in the range of 18-22g. The study was performed according to OECD guideline 423. The animals were divided into 3 groups, with three animals per group. Group I was kept as a vehicle control while Groups II and III served as test groups. All the mice were fasted prior to oral gavage with HC9 for 1-2 h. Individual body weights of animals were taken before dosing. HC9 was administered orally at 1750 and 2000 mg/kg doses of body weight, whereas the control group received distilled water only. Food or water was withheld for 2 h after drug treatment. The animals were closely monitored for initial 4 h after the administration of HC9 and then daily for 14 d to record any signs of toxicity such as tremors, convulsions, salivation, hyperactivity, ataxia, diarrhea, lethargy, sleep, coma, mydriasis, piloerection, gasping and mortality [21-22]. At the end of the study, all the animals were sacrificed to analyze the effect of HC9 on different organs of the mice.

Sub-acute toxicity study

The sub-acute toxicity study of HC9 was performed in Swiss albino mice of either sex having body weights in the range of 18-22 g. All the animals were randomly distributed into 6 different groups (Group I-VI) comprising ten animals (5 males and 5 females) per group (table 1). The sub-acute toxicity study was performed according to OECD guideline 407. Group I served as a vehicle control and received only distilled water. Group III, IV and V received HC9 orally at the doses of 250, 500, 1000 mg/kg, respectively, daily for 28 d. Group II and VI served as satellite control and high dose reversible groups, respectively. Group II received only distilled water and Group VI received 1000 mg/kg doses of HC9 orally for 28 d. The satellite/reversible groups were further observed for next 2 w post-treatment for the reversibility, persistence, or delayed occurrence of toxic effects of HC9 and were sacrificed on 43 d (table 1). Body weight of animals as well as their food and water consumption was recorded weekly throughout the study period. The animals were observed for signs of toxicity and mortality throughout the experimental period. The urine samples and blood samples of all the animals were taken prior to necropsy. After sacrificing the animals, different organs were collected for histopathological analysis.

Urine analysis

During the last week of the study period, urine samples from all the animals were collected and their analysis was done to evaluate various parameters such as appearance, specific gravity, pH, protein, ketone bodies, glucose, nitrite, urobilinogen, leucocytes and occult blood. Dx Urine test 10 (Piramal healthcare Limited, Mumbai) reagent strips were used for urine analysis.

Hematological analysis

The animals were fasted overnight prior to necropsy and blood collection [22]. Blood samples were collected from retro-orbital sinus puncture technique. Different parameters such as hemoglobin (Hb), platelet count (P), lymphocyte count (L), eosinophil count (E), monocyte count (M), basophil count (B), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were estimated.

Biochemical analysis

Biochemical analysis of serum samples was performed to analyze various parameters such as serum creatinine (CREA), triglycerides (TG), total protein (TP), albumin (ALB), total bilirubin (T-BIL), direct

bilirubin (D-BIL), total cholesterol (TCHOL), blood urea, glucose, sodium (Na), potassium (K), serum glutamate oxalo acetate transaminase (SGOT) and alkaline phosphatase (ALP).

Histopathological Analysis

After blood collection, animals were sacrificed and different organs such as brain, kidney, adrenal gland, liver, heart, spleen, thymus, lungs, testis/uterus, and epididymis/ovaries were collected from each mouse to observe histopathological changes in the organs. The absolute weight of all the organs was recorded and the relative organ weight was calculated as:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of mouse on sacrificed day (g)}} \times 100$$

The organs were then fixed in 10% neutral buffered formalin for 18 h at 4 °C and processed by conventional techniques. Paraffin sections were stained with hematoxylin and eosin, following the standard laboratory procedures. The stained sections were examined under the microscope for any cellular damage or change in morphology.

Statistical analysis

Data have been presented as mean±S.D. Statistical analysis was performed with Sigma Stat 3.5 program (Systat Software, Inc.) by using two-way ANOVA with $\alpha=0.05$.

RESULTS

Acute toxicity study

In the acute toxicity study, female Swiss albino mice were orally administered with two different doses (1750 and 2000 mg/kg) of HC9 and monitored for 14 d. The treated mice didn't exhibit any mortality, body weight or behavioral change or toxicity compared to the control group. Morphological features such as fur, skin, eyes, and nose appeared normal. There was no sign of tremors, convulsion, salivation, diarrhea, lethargy or unusual behavior such as self mutilation or walking backward and so forth in the treated mice. There was no change in gait and posture, reactivity to handling, sensory stimuli or change in grip strength in the treatment groups.

Sub-acute toxicity study

General behaviour and mortality

Daily oral administration of HC9 at doses of 250, 500 and 1000 mg/kg for 28 d did not produce any abnormality and toxicity symptoms in mice of either sex (table 1). The treated mice did not show any abnormal clinical signs such as tremors, convulsions, salivation, hyperactivity, ataxia, diarrhea, lethargy, sleep, coma, mydriasis, piloerection and gasping. No mortality was recorded in any mice due to HC9 treatment. In high dose reversible/satellite group, treated with 1000 mg/kg HC9, there were no post-treatment related toxicity symptoms or mortality in any of the mice compared to the satellite control.

Changes in body weight and food consumption

The male and female mice were observed for changes in body weight and food consumption that were recorded weekly during the treatment period as given in table 2 and table 3, respectively. No significant difference ($p>0.05$) in body weights of male and female groups was recorded between control and HC9 treated groups (table 2a and b, respectively). There was non-significant difference ($p>0.05$) within the groups compared to their initial body weights.

Table 1: Study design for sub-acute toxicity of HC9 treatment in Swiss albino mice

Groups	HC9 dose (mg/kg/body weight)	No. of mice	Treatment Days	Sacrificed on day
I (Untreated control)	0	10	28	29
II (Reversal/satellite control)	0	10	28	43
III (Low dose treatment)	250	10	28	29
IV (Inter dose treatment)	500	10	28	29
V (High dose treatment)	1000	10	28	29
VI (High dose reversal/satellite)	1000	10	28	43

Table 2a: Body weights of male Swiss albino mice treated with HC9

Weeks	Body weight change (g)					
	Group I	Group II	Group III	Group IV	Group V	Group VI
0	23.40±0.208	24.40±4.04	25.80±4.72	24.20±3.51	24.00±5.03	24.40±7.02
1	23.00±2.34	26.20±5.93	25.20±3.11	26.80±4.14	24.20±4.49	24.20±2.77
2	29.60±2.60	27.20±4.32	25.80±4.60	30.80±4.94	25.20±4.66	24.80±1.82
3	28.20±4.96	27.40±6.10	25.60±4.03	30.60±5.54	26.20±2.48	23.40±1.67
4	29.80±7.25	27.80±6.68	26.50±3.10	30.20±6.37	26.20±2.48	24.20±1.64
5		25.60±6.38				25.40±1.14
6		27.20±6.64				26.60±1.14

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared to the control mice (p>0.05).

Table 2b: Body weights of female Swiss albino mice treated with HC9

Weeks	Body weight change (g)					
	Group I	Group II	Group III	Group IV	Group V	Group VI
0	22.20±4.08	22.20±3.11	23.00±2.00	22.80±3.03	24.00±5.14	24.40±5.63
1	21.80±3.89	22.00±2.64	23.20±1.92	22.8±2.58	23.80±3.56	21.60±2.70
2	23.80±4.26	22.80±3.70	25.20±1.64	25.2±2.68	23.80±3.42	22.40±2.19
3	24.40±3.50	22.40±3.13	25.20±0.44	24.00±1.58	23.00±3.80	22.00±2.34
4	24.80±2.58	22.20±3.34	24.20±1.30	24.4±1.14	22.40±2.50	22.60±3.04
5		23.40±2.96				24.40±3.20
6		25.00±2.82				25.80±3.11

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05).

There was no significant difference (p>0.05) in the food consumption pattern of HC9 treated groups compared to their respective controls

and also within the groups compared to their initial food consumption of either male or female mice (table 3a and b, respectively).

Table 3a: Food consumption of the male Swiss albino mice treated with HC9

Weeks	Food consumption (g)					
	Group I	Group II	Group III	Group IV	Group V	Group VI
1	5.75±0.59	6.24±1.41	6.30±0.78	5.36±0.83	8.07±1.50	6.05±0.69
2	7.40±0.65	8.50±1.76	6.45±0.96	5.70±0.92	7.41±0.64	6.89±0.46
3	8.00±0.53	6.52±1.45	6.10±0.96	6.65±1.21	6.55±0.62	8.36±0.60
4	6.21±1.51	6.62±1.59	7.00±0.71	5.39±1.14	5.95±0.57	6.72±0.46
5		4.13±1.03				5.77±0.26
6		4.69±1.75				6.94±1.70

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05).

Table 3b: Food consumption of the female Swiss albino mice treated with HC9

Weeks	Food consumption (g)					
	Group I	Group II	Group III	Group IV	Group V	Group VI
1	6.67±1.59	7.29±1.25	6.41±0.71	6.60±1.04	6.36±1.10	6.09±0.43
2	6.98±0.92	7.16±0.68	6.89±0.61	7.08±1.71	6.60±1.13	6.71±1.89
3	7.77±2.29	8.19±1.96	7.45±1.20	7.87±1.21	7.35±1.08	7.18±1.35
4	5.99±0.88	6.31±0.68	6.24±0.52	6.21±0.53	6.02±1.00	6.10±0.99
5		4.68±0.59				5.19±0.62
6		5.00±0.57				5.38±0.65

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05).

Urine analysis

The urinalysis revealed no adverse effects due to HC9 treatment in any mice of either sex compared to the vehicle control group in the 28-day study (Supplementary data S1a-l). The urinalysis parameters such as appearance, blood, nitrate, leukocyte, glucose, pH, protein and specific gravity did not show any significant differences in HC9 treated mice of either sex compared to their respective control groups. All values were in normal range in control and treatment groups of both the sexes (Supplementary data S1a-h). The urinalysis of mice under high dose reversible/satellite groups also didn't

reveal any significant differences compared to satellite control groups of either sex (Supplementary data S1i-l). Thus, the oral administration of HC9 did not affect urine parameters in mice.

Hematological analysis

HC9 did not induce any abnormal changes in hematological parameters such as Hb, MCH, MCHC, PCV, MCV and differential cell counts (P, L, M, E, B) in male and female groups compared to their respective vehicle controls (table 4a and b, respectively). In male groups, all the values, except for eosinophil count (E) of group II

(satellite control group), were in the normal range (table 4a). In female groups, all the values except for eosinophil count (E) of IV group and MCV value in the control group (Group I), were in the normal range (table 4b). The slight differences in MCV or eosinophil (E) values in control or treated mice of male and female groups could be due to some inherent variations in the mice as it

was not reflected in other groups. Interestingly, the mice under high dose reversible/satellite groups didn't show any significant differences in hematological parameters compared to the satellite control groups of either sex. These results suggested that administration of HC9 in mice did not affect the hematological parameters.

Table 4a: Hematological parameters of the male mice treated with HC9

Parameters#	I	II	III	IV	V	VI	Normal range
Hb (g/dl)	12.53±0.81	13±0.46	12.33±0.68	13.33±0.47	13.4±0.2	12.41±0.46	10.2-16.6
P (×103/μl)	320.66±6.03	370.66±15.53	390.00±5.00	343.33±5.13	371.66±8.62	400.00±8.00	140-450×103
L (×103/μl)	61.33±6.66	58.66±8.74	57.33±4.04	61.66±6.11	58.66±8.74	52.23±3.61	55.0-95.0
E (%)	2.66±0.58	4.33±1.53	2.08±1.00	2.00±1.00	2.33±0.58	2.09±1.00	0.0-3.9
M (%)	1.33±0.58	2.66±0.58	1.66±0.58	2.45±0.00	1.33±0.58	2.66±1.53	1.0-4.0
B (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.0-1.0
PCV (%)	39.7±1.44	40.53±1.80	38.1±2.91	41.2±2.1	40.93±0.85	38.33±1.08	39.0-49.0
MCV (fl)	53.86±0.55	53.06±2.06	51.46±1.39	53.09±1.23	50.60±0.44	51.43±1.66	45.4-60.3
MCH (pg)	14.96±0.40	15.16±0.40	14.46±0.42	14.76±0.55	14.43±0.42	15.06±0.21	14.1-19.3
MCHC (g/dl)	30.13±1.28	30.73±0.74	29.50±1.64	31.43±0.12	31.26±0.57	28.60±0.89	30.2-34.2

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05), #Hb: Hemoglobin; P: Platelet counts; L: Lymphocyte count; E: Eosinophil count; M: Monocyte count; B: basophil count; PCV: packed cell volume; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration

Table 4b: Hematological parameters of the female mice treated with HC9

Parameters#	I	II	III	IV	V	VI	Normal range
Hb (g/dl)	12.80±1.10	12.13±0.87	12.33±0.93	12.27±0.57	12.83±0.93	12.83±0.64	10.2-16.6
P (×103/μl)	403.00±3.61	405.67±5.03	400.67±3.79	290.00±4.58	390.00±2.65	353.33±3.06	140-450×103
L (×103/μl)	56.67±3.21	56.33±2.52	56.00±2.65	64.67±4.51	62.67±8.50	55.33±3.79	55.0-95.0
E (%)	1.67±1.15	3.00±2.00	2.00±1.00	4.67±0.58	3.00±1.00	3.33±0.58	0.0-3.9
M (%)	1.67±0.58	3.33±0.58	1.33±0.58	1.67±0.87	2.00±1.00	2.67±0.65	1.0-4.0
B (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.0-1.0
PCV (%)	41.40±2.36	36.93±2.84	38.47±1.72	38.17±2.71	39.97±2.87	39.23±3.23	39.0-49.0
MCV (fl)	63.57±17.61	51.03±2.05	52.03±0.12	52.17±0.40	51.90±1.50	52.97±0.57	45.4-60.3
MCH (pg)	14.90±0.53	14.80±0.95	15.47±0.65	14.77±0.06	15.07±0.68	15.57±0.25	14.1-19.3
MCHC (g/dl)	30.40±1.42	28.87±2.20	30.30±1.04	30.20±0.26	29.80±1.80	29.77±0.92	30.2-34.2

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05), #Hb: Hemoglobin; P: Platelet counts; L: Lymphocyte count; E: Eosinophil count; M: Monocyte count; B: basophil count; PCV: packed cell volume; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Haemoglobin; MCHC: Mean Corpuscular Haemoglobin Concentration

Biochemical parameters of control and HC9 treated male and female groups have been shown in table 5a and 5b, respectively. HC9 did not cause any statistically significant changes (p>0.05) in liver and kidney function tests such as serum SGOT, SGPT, ALP, CREA, TG, TP, ALB, T-BIL, D-BIL, TCHOL, blood urea, glucose as well as levels of electrolytes such as Na and K in treated groups compared to their respective vehicle controls. All the values were within the normal range in both the male and female mice (table 5a and 5b, respectively).

In male control groups, the values of urea, albumin, SGOT and SGPT were found to be outside the normal range. However, differences in SGPT levels between control group I and groups IV and V; and between satellite control group II and group VI (high dose reversible), were found to be non-significant (p>0.05) (table 5a). In female control groups also, the values of urea, albumin and SGPT were found to be outside the normal range. Interestingly, upon HC9 treatment, the values of SGPT decreased in treatment groups compared to their respective controls (table 5b). SGOT/SGPT ratios, indicative of liver function test, were lower in all the treatment groups compared to the respective controls in male and female mice (Tables 5c and d, respectively). Moreover, serum bilirubin levels were within range in HC9 treated male and female mice (tables 5c and d, respectively).

Organ weights

The relative organ weights of the mice treated with HC9 have been shown in table 6. There was no statistically significant difference (p>0.05) found in absolute and relative organ weights of male and

female mice (Supplementary data S2a and b) and table 6a and b, respectively) of the non-reversible and high dose reversible treated groups compared to their respective controls.

Histopathological analysis

Histopathological examinations of different organs of control and HC9 treated mice were done to confirm whether there are any alterations in cell structure [23]. At necropsy, no treatment-related macroscopic changes were observed in any of the treated mice. The analysis of brain, kidney, adrenal gland, liver, heart, spleen, thymus, lungs, testis/uterus and epididymis/ovaries of HC9 treated groups did not reveal any microscopic changes (Supplementary data S3a and b). Some mild cloudy changes observed in kidney sections of male and female groups in both control and treatment groups were considered to be normal as these were also observed in vehicle controls of either sex (Supplementary data S3a and b). Mild swelling was observed in one of the mice in high-dose male group (Group V) and not in other mice within the same group or other treated groups. fig.1a and b show representative histopathological sections of heart, kidney, liver, and lung from male and female mice from each group, respectively. The treatment with HC9 did not show any abnormality in cardiac muscle fibers and glomeruli, tubules and interstitium in kidneys. No abnormality was observed in the hepatocytes and lungs of the treated animals. The histopathological pictures of all the vital organs of other mice from respective groups were similar (data not shown) to that observed in fig.1a and b.

Table 5a: Biochemical findings of the male mice treated with HC9

Parameters#	I	II	III	IV	V	VI	Normal range
TCHOL (mg/dl)	83.33±17.39	73.00±4.58	73.00±25.12	69.33±10.69	76.00±9.17	70.33±4.04	40.0-130.0
GLU (mg/dl)	102.67±37.07	144.00±23.58	85.00±17.69	114.00±8.72	80.67±9.45	122.67±43.75	62.0-175.0
UREA (mmol/l)	42.00±8.19	39.67±5.13	40.00±3.00	38.00±3.0	37.67±3.21	36.67±4.73	8.0-33.0
CREAT(μmol/l)	0.70±0.11	0.53±0.08	0.62±0.12	0.58±0.06	0.51±0.03	0.47±0.05	0.2-0.9
TG (mg/dl)	29.00±1.73	37.67±4.04	30.67±17.21	24.67±11.02	30.67±6.11	31.33±6.66	-
NA (mmol/l)	147.67±6.43	147.67±2.52	153.33±6.43	149.67±1.53	152.67±8.74	148.67±4.51	140.0-160.0
K (mmol/l)	5.57±0.31	6.17±0.21	5.80±0.30	5.73±0.57	6.03±0.49	5.67±0.25	5.0-7.5
TP (g/l)	7.17±0.12	7.17±0.38	7.17±0.38	6.83±0.06	6.60±0.61	6.73±0.12	3.5-7.2
ALB (g/l)	3.57±0.25	3.93±0.15	3.83±0.12	3.60±0.36	3.80±0.44	3.17±0.15	2.5-3.0
T-BIL (μmol/l)	0.40±0.10	0.47±0.05	0.37±0.06	0.37±0.06	0.40±0.10	0.46±0.10	0.0-0.9
D-BIL (μmol/l)	0.22±0.01	0.24±0.04	0.23±0.06	0.21±0.02	0.22±0.03	0.23±0.06	<0.3
SGOT (U/l)	308.33±140.56	305.67±112.59	188.00±8.89	304.67±123.71	270.33±77.73	249.00±88.54	54.0-298.0
SGPT (U/l)	119.67±9.87	176.67±11.59	114.33±33.72	156.33±22.50	188.33±16.86	210.00±40.29	17.0-77.0
ALP (U/l)	47.33±13.65	67.67±4.73	70.00±9.85	67.67±16.04	75.00±15.10	62.67±8.50	35.0-96.0

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05). #TCHOL: Total Cholesterol; Glu: Glucose; UREA: Blood Urea; CREA: Serum Creatinine; TG: Triglycerides; NA: Sodium; K: Potassium; TP: Total Protein; ALB: Albumin; T-BIL: Total Bilirubin; D-BIL: Direct Bilirubin; SGOT: Serum Glutamate Oxaloacetate Transaminase; SGPT: Serum Glutamate Pyruvate Transaminase ALP: Alkaline Phosphatase

Table 5b: Biochemical findings of the female mice treated with HC9

Parameters#	I	II	III	IV	V	VI	Normal range
TCHOL (mg/dl)	80.33±11.02	78.67±10.50	64.00±12.77	81.33±16.07	76.33±5.69	83.33±8.74	40.0-130.0
GLU (mg/dl)	104.33±28.59	114.33±43.66	113.67±43.00	120.67±13.87	96.67±19.50	99.67±13.32	62.0-175.0
UREA (mmol/l)	40.00±3.61	45.00±5.20	34.67±6.66	39.67±2.52	36.33±3.21	37.67±4.16	8.0-33.0
CREAT (μmol/l)	0.49±0.03	0.64±0.11	0.64±0.17	0.41±0.03	0.49±0.03	0.43±0.05	0.2-0.9
TG (mg/dl)	32.33±4.16	35.00±3.61	28.67±7.09	35.67±15.53	33.67±5.03	36.33±5.03	-
NA (mmol/l)	148.67±4.16	148.00±1.00	150.33±4.16	149.33±3.51	149.00±6.24	154.67±1.53	140.0-160.0
K (mmol/l)	6.23±0.40	6.00±0.26	5.50±0.26	5.90±0.40	5.90±0.56	6.17±0.25	5.0-7.5
TP (g/l)	6.20±0.36	7.00±0.10	6.77±0.59	6.83±0.15	6.53±0.40	6.77±0.21	3.5-7.2
ALB (g/l)	3.33±0.45	3.83±0.12	3.77±0.25	3.57±0.29	3.37±0.40	3.50±0.46	2.5-3.0
T-BIL (μmol/l)	0.33±0.06	0.44±0.10	0.39±0.19	0.40±0.08	0.36±0.07	0.40±0.04	0.0-0.9
D-BIL (μmol/l)	0.18±0.09	0.20±0.06	0.21±0.12	0.26±0.05	0.18±0.03	0.22±0.02	<0.3
SGOT (U/l)	298.07±107.05	195.33±42.52	190.67±11.06	302.00±110.96	244.33±23.09	149.00±28.28	54.0-298.0
SGPT (U/l)	237.00±86.28	269.33±64.44	151.00±26.91	173.33±4.51	224.67±31.94	166.33±8.33**	17.0-77.0
ALP (U/l)	64.67±7.77	55.67±24.66	66.00±6.56	71.00±12.12	74.00±0.12	85.67±7.57	35.0-96.0

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05). **Significantly different from the control at p<0.01. #TCHOL: Total Cholesterol; Glu: Glucose; UREA: Blood Urea; CREA: Serum Creatinine; TG: Triglycerides; NA: Sodium; K: Potassium; TP: Total Protein; ALB: Albumin; T-BIL: Total Bilirubin; D-BIL: Direct Bilirubin; SGOT: Serum Glutamate Oxaloacetate Transaminase; SGPT: Serum Glutamate Pyruvate Transaminase; ALP: Alkaline Phosphatase

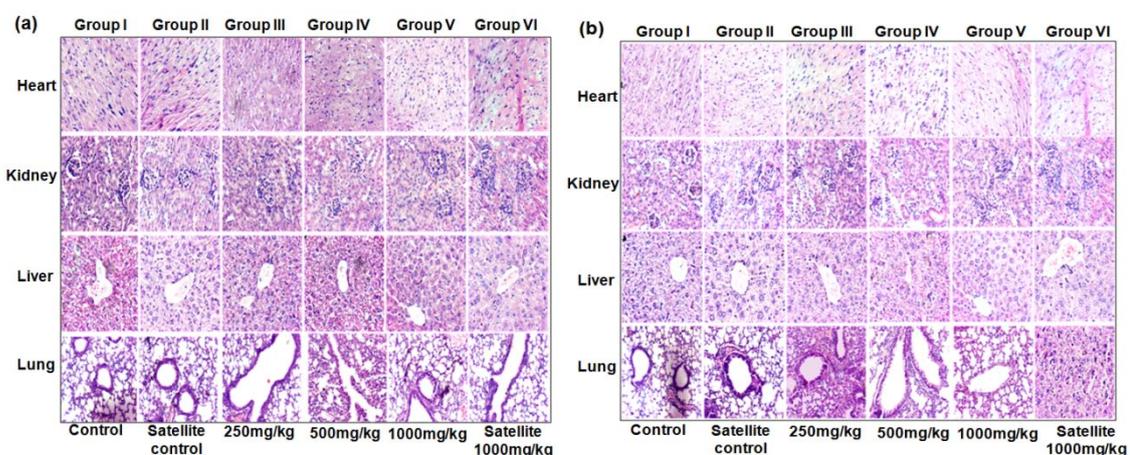


Fig. 1: Histopathology of different tissues isolated from male and female mice observed under microscope

Histological sections of heart, kidney, liver, and lung isolated from (a) male and (b) female mice from each group have been shown. No obvious abnormality in histology was observed in HC9 treated groups at the doses of 250, 500 and 1000 mg/kg compared to the vehicle control groups, as seen under the microscope. No post-treatment related histological changes were observed in any of the organ sections of HC9 treated reversal/satellite mice compared to the satellite control mice.

Table 5c: Liver function test markers of the male mice treated with HC9

Parameters [#]	I	II	III	IV	V	VI
SGOT/SGPT Ratio	2.53±0.92	1.70±0.53	1.74±0.50	2.05±1.08	1.43±0.36	1.17±0.28
T-BIL (µmol/l)	0.40±0.10	0.47±0.05	0.37±0.06	0.37±0.06	0.40±0.10	0.46±0.10
D-BIL (µmol/l)	0.22±0.01	0.24±0.04	0.23±0.06	0.21±0.02	0.22±0.03	0.23±0.06

Values are expressed as mean±standard deviation, n=5.

Table 5d: Liver function test markers of the female mice treated with HC9

Parameters [#]	I	II	III	IV	V	VI
SGOT/SGPT Ratio	1.48±1.07	0.72±0.036	1.28±0.148	1.36±0.308	1.10±0.232	0.85±0.130
T-BIL (µmol/l)	0.43±0.03	0.44±0.10	0.39±0.19	0.40±0.08	0.36±0.07	0.40±0.04
D-BIL (µmol/l)	0.27±0.05	0.20±0.06	0.21±0.12	0.26±0.05	0.18±0.03	0.22±0.02

Values are expressed as mean±standard deviation, n=5.

Table 6a: Relative organ weight of male Swiss albino mice treated with HC9 Relative organ weights of male mice (mg)

Organs	Group I	Group II	Group III	Group IV	Group V	Group VI
Brain	1.91±0.19	2.02±0.87	1.56±0.24	1.56±0.37	1.63±0.28	1.59±0.16
Liver	7.92±0.92	7.32±2.95	6.04±1.20	7.35±2.86	6.47±0.88	5.87±0.32
Kidney	1.51±0.34	1.56±0.65	1.39±0.41	1.50±0.51	1.46±0.27	1.38±0.14
Adrenal	0.03±0.01	0.03±0.01	0.02±0.00	0.03±0.01	0.02±0.00	0.02±0.00
Heart	0.54±0.13	0.64±0.27	0.51±0.06	0.57±0.12	0.57±0.07	0.60±0.10
Thymus	0.23±0.03	0.20±0.04	0.18±0.01	0.19±0.05	0.19±0.04	0.19±0.04
Lung	1.11±0.22	1.13±0.34	0.97±0.27	1.08±0.35	0.68±0.19	0.95±0.18
Spleen	0.57±0.22	0.58±0.12	0.46±0.13	0.46±0.15	0.58±0.19	0.40±0.07
Testis	0.77±0.23	0.93±0.44	0.72±0.09	0.59±0.09	0.71±0.08	0.88±0.08
Epididymids	0.27±0.06	0.32±0.12	0.25±0.03	0.28±0.10	0.27±0.10	0.35±0.02

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05).

Table 6b: Relative organ weight of female Swiss albino mice treated with HC9 Relative organ weights of female mice (mg)

Organs	Group I	Group II	Group III	Group IV	Group V	Group VI
Brain	1.83±0.36	1.64±0.32	1.73±0.06	1.62±0.04	1.83±0.06	1.69±0.13
Liver	7.16±0.39	5.61±1.01	6.69±0.86	6.71±0.20	6.13±0.61	5.46±0.42
Kidney	1.20±0.21	1.18±0.26	1.05±0.08	1.15±0.14	1.10±0.12	1.11±0.21
Adrenal	0.02±0.00	0.02±0.00	0.03±0.01	0.03±0.01	0.03±0.01	0.03±0.01
Heart	0.55±0.11	0.57±0.09	0.56±0.12	0.53±0.03	0.54±0.09	0.55±0.02
Thymus	0.30±0.16	0.29±0.07	0.23±0.04	0.27±0.05	0.28±0.04	0.27±0.06
Lung	1.31±0.10	1.21±0.59	1.23±0.16	1.29±0.26	1.27±0.19	1.18±0.12
Spleen	0.49±0.12	0.48±0.05	0.45±0.10	0.46±0.05	0.42±0.12	0.49±0.06
Ovaries	0.08±0.03	0.06±0.01	0.07±0.03	0.09±0.02	0.08±0.00	0.06±0.00
Uterus	0.33±0.06	0.31±0.07	0.30±0.03	0.25±0.02	0.32±0.05	0.28±0.07

Values are expressed as mean±standard deviation, n=5. HC9 treated groups showed non-significant differences as compared with control mice (p>0.05).

DISCUSSION

Herbal medicines have gained increasing global interest due to their safety as well as potential to act against various diseases including cancer [1-7]. Extensive research is being focused towards the traditional and herbal medicines as main sources of chemopreventive drugs [8-14]. Many herbal remedies have been reported to possess anti-cancer activities and some of them have been used by cancer patients for many years [11-14]. However, the safety and toxicity assessment of herbal medicines is rarely done before their human consumption as they are considered to be inherently safe [7]. The clinical use of herbal drugs without adequate scientific evidence has raised concerns regarding their toxicity status [24]. Thus, toxicity evaluation of herbal medicines is being carried out in various experimental animal models to predict their safety and for selecting a 'safe' dose regimen for future human use [23, 25].

HC9 is a polyherbal composition of nine medicinal herbs, each one reported to have pharmacological action including anticancer, anti-inflammatory and immunomodulatory activity. We have previously

reported that HC9 exhibited significant antioxidant potential and cytotoxic activity in breast cancer cell lines [8]. Moreover, it also exhibited significant anticancer and immunomodulatory activity (communicated). We have standardized the formulation with respect to its marker compounds [20]. Since, HC9 is effective against the breast cancer cell lines, so we wanted to evaluate its toxicity for possible therapeutic application. In the present work, the acute and subacute toxicity of HC9 was mainly performed to identify a safe dose regimen and the therapeutic index of the drug for its possible future use. Sub-acute toxicity study is a widely accepted test to evaluate any possible health hazards due to treatment with the drugs [24, 26]. HC9 didn't reveal any treatment related adverse effects on vital functions of the mice that include cardiovascular, central nervous and respiratory systems. Interestingly, 28-day sub-acute toxicity study did not reveal any adverse effects of HC9 on body weights, food consumption, urinalysis, hematology, serum biochemistry, gross pathology, organ weights or histopathology. HC9 had no gross adverse effect on the hematological parameters in mice. The hematological system is an important indicator of the

physiological and pathological status of animals or humans [27]. It is highly sensitive to toxic compounds and small changes in the hematological system could have higher predictive value for drug associated toxicity [27]. All the hematological parameters in the treatment groups were within the normal range, except for eosinophil count (E) of 500 mg/kg treatment group of female mice. However, it could be due to some inherent variations in the mice as it was not reflected in other groups. Besides, other related parameters such as Hb, MCH, MCHC, PCV, MCV, P, L, M and B of the mice in the same group were normal. Moreover, the mice under high dose reversible/satellite groups didn't show any significant difference in hematological parameters compared to the satellite control groups of either sex. Therefore, it could be concluded that HC9 was safe for the mice.

HC9 did not cause any significant change in biochemical parameters for hepatic and renal functions such as SGOT, ALP, triglycerides, protein, albumin, bilirubin and cholesterol. Clinical biochemistry is mainly performed to evaluate the effect of drugs on hepatic and renal functions, serum electrolytes as well as glucose and total cholesterol levels [28]. Though the values of urea, albumin, SGOT and SGPT of control and treated groups were found to be outside the normal range, upon HC9 treatment, the values of urea, albumin and SGOT were found to decrease in treatment groups compared to their respective controls. These results suggest that control mice may have some inherent problem with liver function. Hepatic biochemical parameters provide valuable information on the status of the liver in terms of its functionality, cellular integrity, synthesis and its link with biliary tract [29]. Enzymes such as SGOT and ALP are well-known indicators of liver function that predict drug related toxicity [30]. The ratio of SGOT/SGPT, also known as De Ritis Ratio, is an indicative of liver function test (De Ritis F *et al.*, 1957). If the ratio is high, it is predictive of liver related complications. Along with SGOT/SGPT ratio, serum bilirubin levels are also an important indicator of liver function test (Reuben A, 2004). Since, liver is the major site of synthesis of cholesterol, protein and albumin as well as for cholesterol disposal or degradation, any changes in these parameters could be suggestive of liver dysfunction that may be due to drug toxicity [30-31]. Our results showed high SGPT levels at 500 and 1000 mg/kg doses in male mice than the control group. However, SGOT/SGPT ratios, were lower in all the treatment groups compared to the respective controls in male and female mice (table 5.7a-b). Interestingly, serum bilirubin levels were within range in HC9 treated mice of either sex. Besides the above facts, macroscopic observation of organs, organ weights and histological examinations of the liver sections together with asymptomatic clinical and behavioral signs did not reveal any signs of toxicity upon HC9 treatment. All together, the data suggested that HC9 did not affect the liver function of mice.

HC9 did not affect the serum levels of blood urea and creatinine in any mice of either sex at any dose compared to their respective controls. Blood urea and creatinine are important markers of renal toxicity [24, 32-34]. There are several reports of kidney toxicity related to the use of phytotherapeutic drugs, as kidneys eliminate many drugs and their metabolites [29]. Our data showed that HC9 does not alter the kidney function of mice.

Organ weight is an important index of physiological and pathological status of animals. HC9 didn't affect the absolute and relative organ weights of treated mice of either sex. Histological examinations also supported the conclusions from clinical biochemistry studies that oral administration of HC9 did not induce any renal or liver damage even at higher doses (1000 mg/kg). HC9 did not induce any pathological changes in the heart, brain, adrenal gland, thymus, lung, spleen and reproductive organs such as testes/uterus and epididymes/ovaries of the mice. Thus, the polyherbal formulation, HC9, could be considered to be non-toxic and safe for its future applications.

CONCLUSION

Medicinal plants have played an important role in world health, as they are potential sources of new therapeutic agents. Thus, it becomes important to evaluate their apparent toxicity. The present study showed that HC9, a PHF, didn't induce any apparent toxicity in

mice even at higher doses. Thereby, suggesting its possible therapeutic application.

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CONFLICT OF INTERESTS

The authors have declared that there is no conflict of interest

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